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14. ABSTRACT

Telomerase is reactivated in almost all human breast cancers; loss of telomeric protection usually leads to genomic instability. This proposal is to study telomerase reactivation and telomere protection in newly immortalized human mammary epithelial cells (HMEC) that retain wild type p53 function, and to determine if these cells may be especially sensitive to therapies that target telomerase activity and telomere protection. Prior work showed that p53 can suppress most, but not all, telomerase expression in newly immortal p53+ HMEC lines until telomeres become extremely short, when an unknown mechanism (termed conversion) relieves this repression. We hypothesized that the observed upregulation of cyclin-dependent kinase inhibitor p57 might protect cells with critically shortened telomeres by inhibiting growth until there is sufficient telomerase to protect the telomeric ends. However, in the past year we observed that inhibition of p57 produced a result similar to that seen when telomerase was inhibited, and did not lead to mitotic failure. We are currently addressing the questions: (1) What is the role of upregulated p57 during conversion; (2) Can inhibition of telomerase and/or p57 efficiently kill these cells; (3) How does p53 regulate telomerase activity in newly immortal HMEC lines.

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INTRODUCTION:

Acquisition of an immortal potential is considered crucial for human carcinogenesis, in order for a single cell to accumulate the multiple errors necessary for malignancy. In human carcinomas, attaining immortality is associated with reactivation of telomerase activity, which maintains the telomeric ends. Loss of telomeric protection usually leads to telomeric associations and genomic instability. In the continuing absence of telomerase activity, cell death or irreversible growth arrest will ensue. This proposal is designed to study the mechanisms involved in telomerase reactivation and telomere protection in newly immortalized human mammary epithelial cells (HMEC) that retain wild type p53 function, and to determine if these cells may be especially sensitive to killing by agents that target telomerase activity and telomere protection. Prior work showed that p53 can suppress most, but not all, telomerase expression in newly immortal p53+ HMEC lines, leading to a gradual erosion in telomere length until telomeres become extremely short (mean TRF $\leq$3 kb) (Stampfer et al., 2003, Stampfer et al., 1997). At that point, most growth stops, and levels of the cyclin-dependent kinase inhibitor p57 are elevated (Nijjar et al., 1999). An unknown mechanism (termed conversion) then gradually relieves this p53-mediated repression, although all other tested p53 functions are retained (Stampfer et al., 2003, Stampfer et al., 1997), leading to telomerase reactivation, stabilization of telomere length, and resumption of good growth; levels of p57 are gradually reduced concordant with these conversion-associated changes. Based on preliminary data we hypothesized that p57 might be acting to protect these critically shortened telomeres by inhibiting growth until there was sufficient telomerase reactivation to protect the telomeric ends. Therefore, our aims were to (1) Test whether the very low levels of telomerase present in newly immortal p53+ HMEC lines preferentially maintain the shortest telomere ends, until the conversion associated relief of p53-mediated suppression of telomerase activity occurs. (2) Test whether the low telomerase activity, along with the elevated p57 expression, suppress the genomic instability that occurs prior to immortalization (Romanov et al, 2001, Chin et al., 2004), and whether inhibition of telomerase activity and p57 function might efficiently kill the newly immortal cells. (3) Determine how p53 regulates telomerase activity in newly immortal HMEC lines. Some surprising results in the past year have led us to modify our hypotheses. Inhibition of p57 function did not affect the morphology of the cells arrested with critically short telomeres, nor did this population show an accelerated death as would be expected if the elevated levels of p57 were preventing proliferation that could lead to mitotic catastrophe. Rather, inhibition of p57 function led to growth arrest occurring at an earlier passage than control cultures, with no appearance of cells that had reactivated telomerase and thereby initiated the conversion process – a result similar to what was observed when telomerase activity was inhibited.

BODY:

Due to a delay in obtaining the human use approval for this project, work did not fully commence until 6/6/05. Some parts of this proposal have not been initiated due to a delay in obtaining proprietary material from Geron Corporation, the telomerase-inhibiting GRN163L oligonucleotide (and control sequence). All paperwork for this MTA has now been completed and we anticipate receiving this material in the coming month.

Our prior work indicated that p53-dependent p57 expression is elevated in pre-conversion populations of newly immortal p53+ HMEC lines. In the p53+ immortally transformed line 184A1, we have shown that p57 elevation in the cycling population is coincident with the time when the mean TRF declines to <3 kb and most cells enter a growth arrest. p57 levels then gradually decrease as telomerase activity gradually increases, and the population gradually resumes proliferation. In a preliminary experiment, telomerase activity was inhibited in pre-conversion 184A1 using a dominant negative construct against hTERT, GRN385, transduced into 184A1 at passage 12. Treated cultures showed earlier growth arrest and p57 elevation than controls (passage 14 vs. passage 17-18). The morphology of the growth-arrested cells was similar in both cases. However, the control

cultures contained cells with slow growth that gradually reactivated telomerase and underwent conversion, whereas all the GRN385-transduced cells showed a very gradual loss of viability over 1-2 months. We had postulated that the elevated levels of p57 might be involved in restraining proliferation at this arrest until the p53 repression of telomerase was relieved and sufficient telomerase was reactivated to protect the telomeric ends and the cells' viability. In the absence of reactivated telomerase, ongoing proliferation with such critically short telomeres (< 3 kb) would lead to mitotic failure.

In the past year, we examined the effect of inhibiting p57 on pre-conversion 184A1. Retroviral vectors containing shRNA specific to p57, and control oligos (see Figure 1) were transduced into pre-conversion 184A1. Our hypothesis predicted that the p57sh-transduced cells would exhibit a less complete arrest when telomeres became critically shortened, with cells continuing to proliferate and therefore dying more rapidly. However, our results from several experiments showed that the p57-transduced cultures behaved very similar to what we observed in the GRN385-transduced cultures. Cells showed an earlier growth arrest and a very gradual loss of viability, while displaying a morphology similar to the growth-arrested controls (Figures 1-2). Growth-arrested p57sh-transduced populations showed evidence of a p53 response, with increased levels of expression of p21 and p53, and increased p53-Ser15 and Chk2-Thr68 phosphorylation.

The mechanism responsible for this result is not immediately obvious. On the surface, it would appear that inhibition of p57 function is having the same effect on cell growth as inhibition of telomerase activity, and that the growth arrest observed at critically short telomere lengths can be mediated by p53 alone. The latter possibility is not surprising, since we have observed that p53 is responsible for a similar growth arrest in senescent HMEC when the mean TRF of the population declines below 5 kb. In the senescent population, telomere length would be assumed to be heterogeneous, so that one critically shortened telomere could induce a p53-mediated DNA damage response. In the situation with the pre-conversion 184A1, we hypothesize that the telomere lengths are more homogeneous because the low level of telomerase normally present, while not sufficient to preclude ongoing telomere erosion, may preferentially maintain the shortest telomeres lengths; therefore telomere dysfunction is not seen until a shorter mean TRF length of < 3 kb.

To address whether inhibition of p57 function is interfering with telomerase activity or reactivation, we are currently testing successive passage levels of p57sh and control transduced pre-conversion 184A1 populations for mean TRF length, telomerase activity, and p53/DNA damage responses. These data should let us know if inhibition of p57 is correlated with more rapid shortening of the mean TRF, and if induction of a p53 response correlates with attainment of critically shortened telomeres. We will also carefully repeat our preliminary experiment on the effects of telomerase inhibition by exposing the same populations of pre-conversion 184A1 to GRN385 and GRN163L, and assaying for telomere length and p53/DNA damage responses. We will also assay the effect of simultaneously inhibiting telomerase activity and p57 function. Our future studies will depend upon the results from these experiments. If the effect of p57 inhibition mirrors that seen with telomerase inhibition, we will need to consider and explore possible mechanistic explanations for how the presence of p57 may play a role in permitting telomerase activity and reactivation in newly immortal p53+ HMEC lines. It is possible that the p53-dependent p57 expression may be necessary to relieve the p53-mediated repression of telomerase activity. In any case, these results support a potential clinical use for inhibition of p57 and/or telomerase in early stage p53+ breast carcinogenesis, since inhibition of p57 led to premature cell growth arrest and death.

A second aim of this proposal is to understand how p53 acts to suppress telomerase activity in newly immortal p53+ HMEC lines. For these studies we have utilized a genetic suppressor element (GSE22) that acts as a dominant negative inhibitor of p53 tetramer formation (Stampfer et al. 2003). When GSE22 was transduced into a newly immortalized p53+ line, 184A1, levels of hTERT mRNA and telomerase activity quickly

increased, and telomere length was stabilized (Stampfer et al, 2003). As a first step in determining the mechanism of p53 repression of hTERT, the timing of telomerase reactivation in GSE22-transduced 184A1 was measured. Telomerase activity was up-regulated within 96 hours of p53 inactivation (Figure 3), suggesting that the repression of telomerase by endogenous p53 is indirect. It is conceivable, though unlikely, that p53 could be acting directly to induce a long-lived repressor of telomerase transcription, so that the effect of p53 inactivation could take time to manifest. We do believe that p53 is acting on hTERT transcription because of our previously demonstrated effect of GSE22 on hTERT expression (Stampfer et al., 2003) and because the hTERT promoter showed increased activity in GSE22-transduced cells (Figure 4). Future studies will identify the repressive regulatory element within the first 179 bp of the hTERT promoter. Transcription factors Sp1 and Sp3, and histone deacetylases, may be implicated in telomerase-dependent conversion to full immortality of 184A1. Preliminary studies (Figure 5) suggest that the histone deacetylase inhibitor trichostatin A can increase telomerase activity in pre-conversion 184A1, implying a possible role of chromatin conformation in the suppression of telomerase in this population.

In related studies, we have collaborated with Steven Haney at Wyeth, Cambridge MA, to perform expression array profiles of p53(+) vs. p53(-) immortalized HMEC lines, and finite vs. immortal HMEC. A manuscript is close to submission on this work, which indicates that greatest changes in expression occur in the transition from finite strains to non-malignant immortal lines (such as the 184A1 line), and that immortalized non-malignant lines like 184A1 resemble cells found in DCIS in vivo. These studies support the use of this cell line as a model of early stage human breast carcinogenesis, and the testing of effects of potential therapeutics on these cells. The data also reinforce our concern that immortally transformed non-malignant lines should not be referred to (as is commonly done) as normal, since they likely have already acquired the rate-limiting steps in carcinogenesis.

KEY RESEARCH ACCOMPLISHMENTS:

• Shown that inhibition of p57 in pre-conversion p53+ immortalized HMEC produces an effect similar to inhibition of telomerase activity, i.e., earlier growth arrest, suppression of conversion, and eventual cell death.

• Shown that p53-mediated inhibition of telomerase activity in pre-conversion immortalized HMEC is a transcriptional effect that may involve chromatin conformation.

• Shown that p53(+) immortalized HMEC lines have a gene expression profile that resembles cells from DCIS in vivo.

REPORTABLE OUTCOMES:

Mechanisms of hTERT up-regulation during immortalization of human mammary epithelial cells, Ekaterina Bassett, James C. Garbe, Tarlochan Nijjar, Martha R. Stampfer, and Paul Yaswen, abstract, AACR Annual Meeting, Washington DC, April 2006

Yizheng Li, Jing Pan, Jian-Liang Li, Jee Hyung Lee, Chris Tunkey, Katie Saraf, James Garbe, Scott Jelinsky, Martha Stampfer and Steven A. Haney Transcriptional Changes Associated with Breast Cancer Occur as Normal Human Mammary Epithelial Cells Overcome Senescence Barriers and Become Immortalized, in prep.

CONCLUSIONS:

Telomerase is reactivated in almost all human breast cancers; the immortal potential conferred by telomerase is thought to be crucial for malignant progression. Expression of hTERT, the catalytic subunit of human telomerase, is the rate-limiting component of telomerase activity.

Our research has uncovered novel steps involving telomerase repression and reactivation during the immortalization of p53+ HMEC. These studies have shown that newly immortal p53+ HMEC possess exceedingly short telomeres, yet are protected from the widespread genomic instability that could lead to cell death. We hypothesize that the short telomeres result from the observed p53-mediated repression of most telomerase activity, while the protection results from low levels of telomerase present preferentially maintaining the shortest telomeres until the conversion process relieves the p53-mediated repression of telomerase. Our current proposal seeks to expand upon this data, both in terms of understanding the basic mechanisms regulating telomerase expression in these cells, and to determine if such cells, with exceedingly short telomeres, could be especially vulnerable to therapies that target telomerase activity and/or propel cells into catastrophic genomic instability.

Our results from the past year support the possibility that newly immortal p53+ HMEC may be especially vulnerable to specific molecular manipulation. Inhibiting the function of the cyclin-dependent kinase inhibitor p57, as well as inhibition of telomerase activity, led to premature growth arrest and death of the entire newly immortal p53+ population, with no cells showing telomerase reactivation and conversion to full immortality. More studies are needed to understand these phenomena at a mechanistic level, and to consider translational studies of clinical relevance for early stage breast carcinogenesis. In particular, we want to examine the relationship of p53, p57 and hTERT expression, in conjunction with critically short telomeres inducing a DNA damage response, growth arrest or death, and/or genomic instability. We will explore the possibility that p53-dependent expression of p57 may be part of a feedback mechanism necessary for relief of the p53-mediated repression of telomerase activity in these HMEC lines.

More studies are also planned to understand the mechanisms by which p53 suppresses the transcription of hTERT in these newly immortal HMEC populations, including investigation of how transcriptional regulators Sp1, Sp3, and p21 may be involved in p53-mediated regulation of the hTERT promoter.

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Nijjar, T, Bassett, E, Garbe, J, Takenaka, Y, Stampfer, MR, Gilley, D, Yaswen, P, Accumulation and altered localization of telomere-associated protein TRF2 in immortally transformed and tumor-derived human breast cells, Oncogene:24, 3369–3376, 2005.

Romanov, SR, Kozakiewicz, K, Holst, CR, Stampfer, MR, Haupt, LM, Tlsty, TD, Normal human mammary epithelial cells spontaneously escape senescence and acquire genomic changes, Nature 409:633-637, 2001.

Stampfer, M, Garbe, J, Nijjar, T, Wigington, D, Swisshelm, K, Yaswen, P, Loss of p53 function accelerates acquisition of telomerase activity in indefinite lifespan human mammary epithelial cell lines, Oncogene 22:5238-5251, 2003.

Stampfer, MR, Bodnar, A, Garbe, J, Wong, M, Pan, A, Villeponteau, B, Yaswen, P, Gradual phenotypic conversion associated with immortalization of cultured human mammary epithelial cells, Mol Biol Cell 8:2391-2405, 1997.

APPENDICES:

N/A

SUPPORTING DATA:

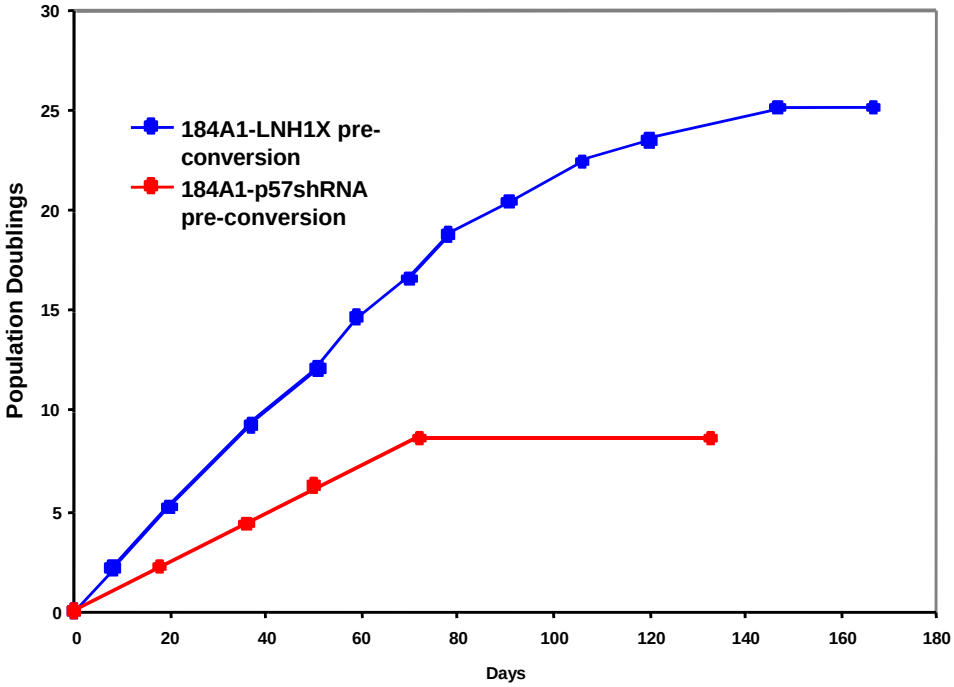

Figure 1. Decreased p57 expression in pre-conversion 184A1 leads to an earlier growth arrest. 184A1 at passage 13 was transduced with retroviral constructs containing shRNA to p57 or empty virus. Treated cultures showed a much earlier growth arrest compared to controls. The retroviral vector pLNH1X was derived from the pLNSX vector by replacing the SV40 promoter with the human histone H1 promoter. To generate the pLNH1X-p57shRNA vector, two synthetic oligonucleotides, si-p57-Forward (GATCCCCGAGCTGAACGCCGAGGACCTTCAAGAGAGGTCCTCGGCGTTCAGCTCTTTTTGGAAA), and si-p57-Reverse (AGCTTTTCCAAAAAGAGCTGAACGCCGAGGACCTCTCTTGAAGGTCCTCGGCGTTCAGCTCGGG), were annealed and ligated into pLNH1X vector. Retroviral stocks were generated by transient co-transfection of the recombinant retroviral plasmid along with a plasmid encoding packaging functions into the 293 cell line. Pre-conversion 184A1 were infected with viral supernatants in the presence of polybrene, and selected in 100μg/mL G418.

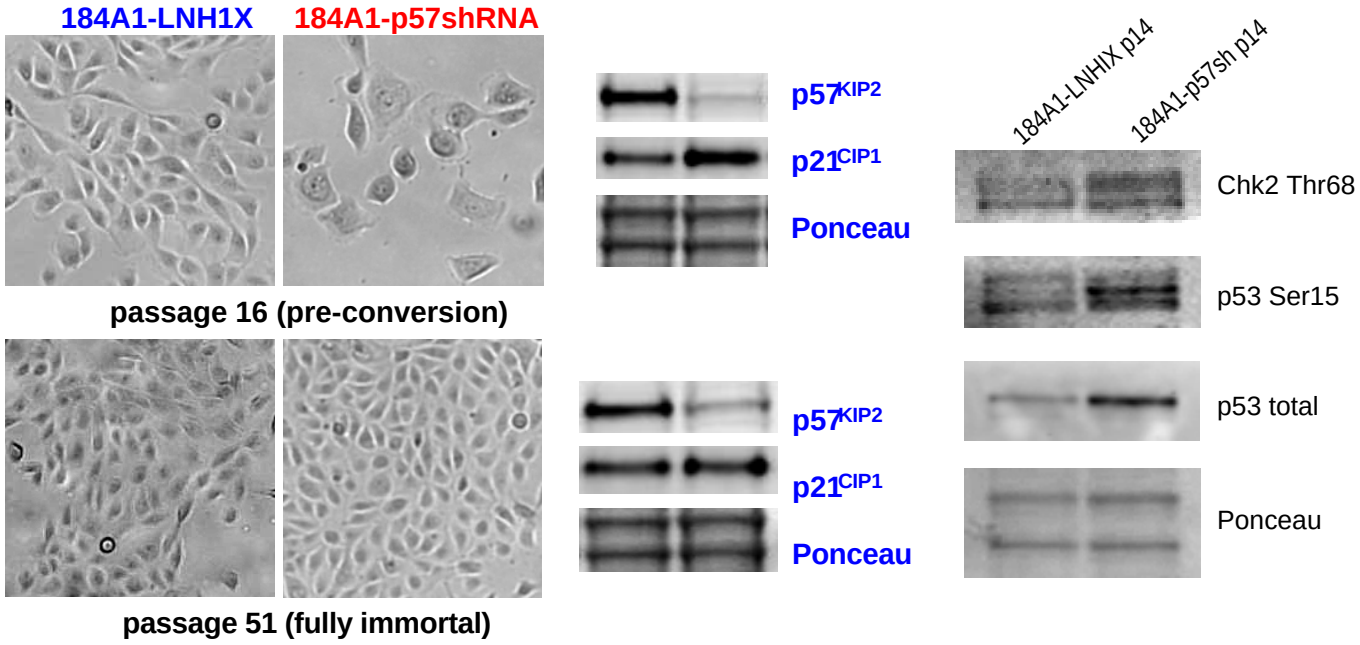

Figure 2. Decreased p57 expression is demonstrated in pre-conversion and fully immortal 184A1 transduced with p57sh, but leads to an earlier growth arrest only in the pre-conversion population. Evidence of p53 activation/DNA damage response is seen at early passages in the p57sh-transduced population.

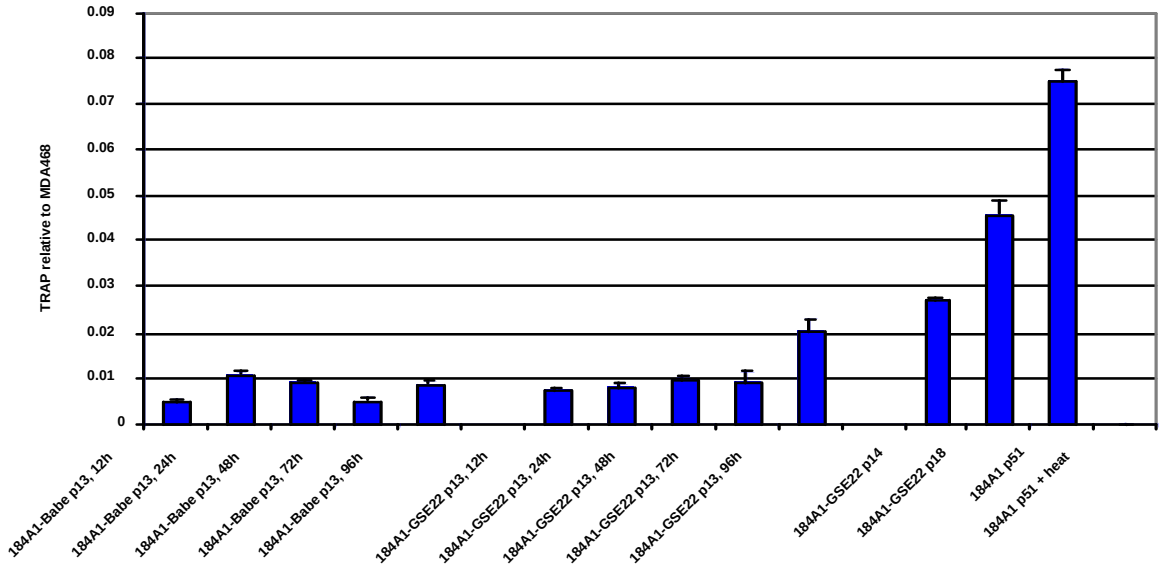

Figure 3. Telomerase activity in pre-conversion 184A1 is up-regulated within 96 hours of GSE22 transduction. 184A1 were infected with high-titer retroviruses carrying either the empty pBabe vector, or the pBabe-GSE22 construct at passage 13. At the indicated time points, ranging from 12h to 96h, subclonfluent cultures were collected by trypsinization, and the cell pellets were processed for real-time TRAP assay using the Quantitative Telomerase Detection Kit (Allied Biotech), following the manufacturer's protocols. 184A1-GSE22 at the later passages, and fully immortal 184A1 cells at passage 51, were used as positive controls for increased telomerase activity.

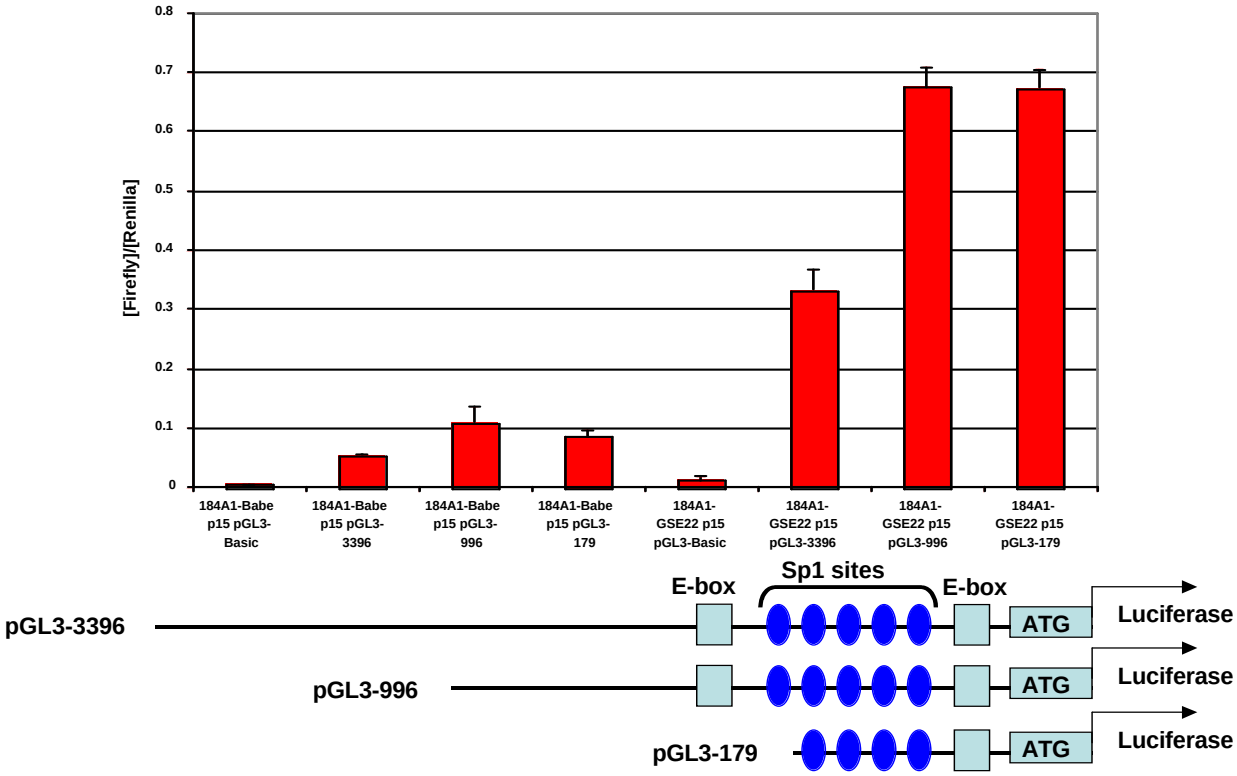

Figure 4. hTERT promoter activity of the firefly luciferase reporter in pre-conversion 184A1 with and without GSE22 inactivation of p53 function. Schematics were adapted from Won *et al.*, *JBC* 277:38230, 2002, and the firefly luciferase hTERT reporter plasmids were obtained from Dr. Tae Kook Kim, Advanced Institute of Science and Technology, Daejeon, South Korea. The firefly luciferase reporter plasmids were co-transfected into 184A1-Babe and 184A1-GSE22 cells at a 1:1 ratio with the promoterless Renilla reporter plasmid (pRL-Null). Following a 48h incubation, subclonfluent cultures were lysed and assayed using the Dual-Luciferase Reporter Assay (Promega). The results are reported as ratios of the firefly reporter luminescence to the Renilla reporter luminescence.

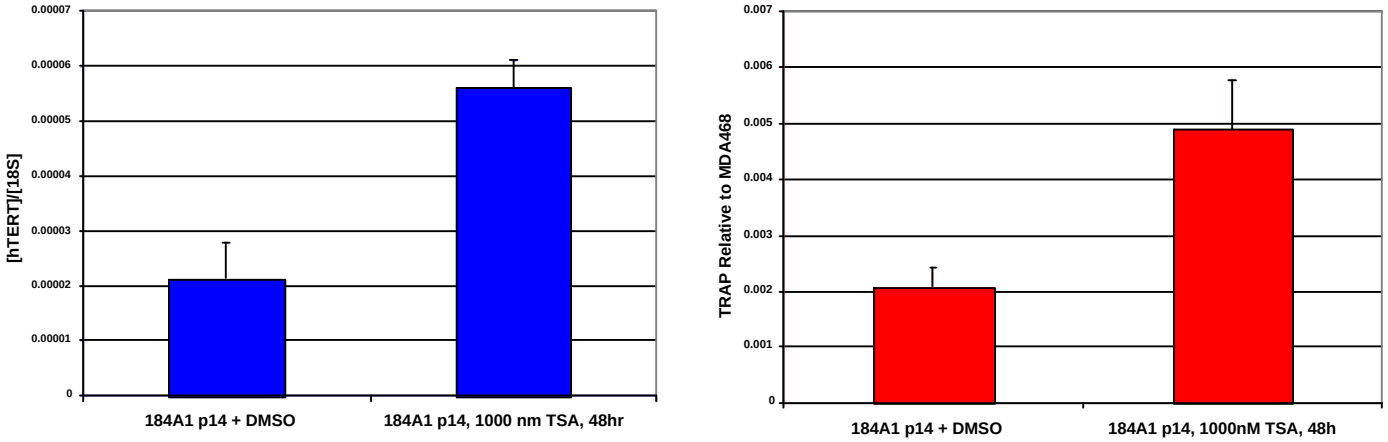

Figure 5. hTERT expression and telomerase activity in pre-conversion 184A1 are induced by the histone deacetylase inhibitor, trichostatin A. 184A1 at passage 14 were treated with either 1000 nM trichostatin A, or DMSO solvent. Following a 48h incubation, parallel subconfluent cultures were harvested for either real-time TRAP assay (Allied Biotech), or for RNA. RNA was isolated using RNeasy Mini Kit (Qiagen), and converted to cDNA using iScript cDNA Synthesis Kit (BioRad). The levels of hTERT expression were determined by real-time PCR, using iQ SYBR Green probe (BioRad). Specific primers were used to amplify hTERT mRNA as a full-length product. Quantification of the product was performed by comparing the levels of hTERT expression to the levels of expression of a housekeeping gene, 18S ribosomal RNA.